# ISOLATION AND CHARACTERIZATION OF CELLULASE PRODUCING MICROBES FROM RUMINANTS FOR BIOREMEDIATION OF ORGANIC WASTES TO OBTAIN USEFUL BYPRODUCT

**SYNOPSIS**

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## INTRODUCTION

Ruminants in India are mainly fed lignocellulosic biomass which is rich in cellulose, hemicelluloses, starch, pectin, sugars, lignin (phenolic component), protein and different types of small quantities of oil. Rumen microbes live in symbiotic relationship with ruminants. Microbes provide digested and broken down food to the bovines and in return are provided with food and anaerobic conditions. The ability of microbes to breakdown complex biomasses efficiently to provide nutrients to the ruminants makes them a useful source for breakdown of organic wastes.

The first compartment of the ruminant's stomach, i.e., rumen comprises an ecosystem populated with variety of organisms that include bacteria (*Ruminococcus* sp., *Prevotella* sp., *Butyrivibrio* sp.,*Clostridium* sp., etc), protozoa (*Diplodinium* sp., *Isotricha* sp., *Oscillospira* sp., etc), fungi (*Neocallimastix* sp., *Sphaeromonas* sp., etc) and bacteriophage (TM7). Due to its anaerobic environment, a temperature of about 39˚C and pH between 6-6.9, the rumen encourages the growth of these diverse microbial populations which are responsible for the breakdown and digestion of food resulting in the production sugars and acids and their further utilization for vitamin synthesis and production of waste products such as methane. Ruminal microbes are highly responsive to the feeding habits, age and health of the ruminants (Kamra, 2005).

Now a days, when the Government of India is moving ahead with the notion 'CLEAN INDIA', the present work plan also deals with idea of cleaning India by the process of Bioremediation of different biomass wastes and their efficient utilization by obtaining useful byproducts like alcohol. Biomass has got energy potential of about 66,000MW of which only 900MW is usually utilized and a large portion of this unused biomass can find application in various fields (Javalkar & Alam, 2013).

There are a variety of biomass wastes like forest waste (wood), agricultural wastes (sugarcane bagasse, petha waste, corn cob, rice husk), industrial wastes (paper), municipal solid waste, etc. (Kumar et. al., 2009). Of the biomass, cellulose comprises the 40% content, hemicellulose is 24%, and lignin is 25% approximately, on percent dry basis (Corcoran *et al.*, 2008*)*.Plant materials are not

so easily digested by animals because of the presence of the ligno-cellulosic matrix which evenly distributes the stresses among the re-enforcing elements thus giving it a composite nature (Mtui, 2009).

Various biomass conversion techniques have been proposed like direct chemical or thermochemical methods (acid hydrolysis and pyrolysis), and biological ones (application of Cellulase enzymes or utilizing microbes that breakdown cellulose). Inspite of several limitations chemical methods are more economical compared to biological ones as cellulase enzymes is expensive due to its low yield in microbes (Bakare *et al.,* 2005). However lignocellulosic biomass can be broken down by microbial hydrolysis. This involves the pretreatment of the biomass to break down the crystalline structure by removal of phenolic lignin and thus exposure of the polysaccharide cellulose and hemicelluloses. Further saccharification of the polysaccharides by microbial enzymatic hydrolysis into monomers like glucose, xylose, and arabinose along with some decomposition product furfural, hydroxymethylfurfural, formic acid, levulinic acid, etc., is required. The ideal hydrolysis time length is very crucial where the cellulose is degraded initially to monomers (six carbon sugar and five-carbon sugar), and on further hydrolysis to acids. And finally, the Fermentation of the obtained monomers under specified condition is carried out to obtain useful byproduct like ethanol (Kumar *et al.,* 2009).

Cellulose, the most abundant polymer in nature is a linear polysaccharide of up to 15000 D-glucose residues having β (1-4) glycosidic linkage and often occurs in combination with other materials like lignin and hemicelluloses (Verma *et al.,* 2012). It may undergo either Cellulolysis (incomplete break down in smaller polysaccharides called cellodextrins) or Hydrolysis (complete breakdown into glucose) (Verma *et al.,* 2012). Abundant availability of the cellulose makes it an attractive raw material through most of the cellulosic wastes are often disposed off by biomass burning (Gupta *et al*, 2012)

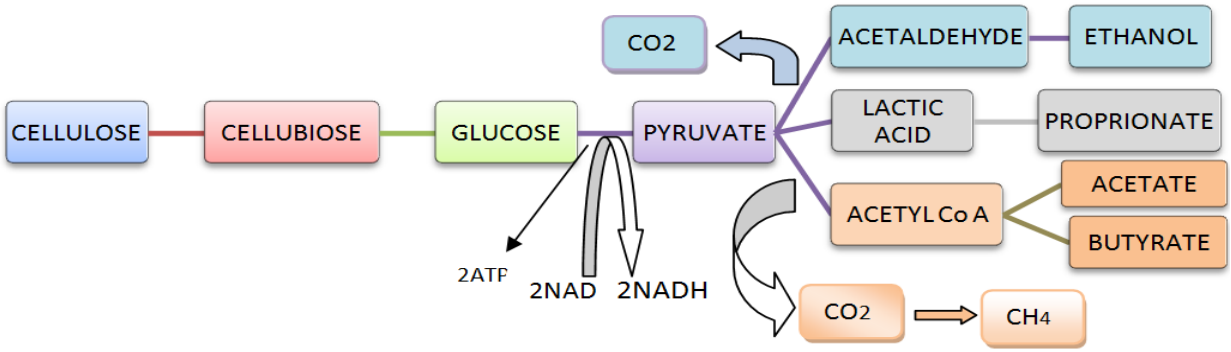
Cellulose can be broken down by the enzyme Cellulase. 4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase also called Cellulase enzyme (EC 3.2.1.4), can be classified into 5 types on the basis of the reaction

catalyzed by them– Endocellulase, Exocellulase, Cellobiase / β-glucosidases, Oxidative Cellulase, Cellulose phosphorylases (Khan *et al.*, 2011). Of these the first three have been studied elaborately.

Cellulase is today an important industrial enzyme. Its applications include: - "bio polishing" of fabrics such as production of the stonewashed look of denims, and in household laundry detergents to improve fabric softness and brightness, as animal feeds to improve nutritional quality and digestibility, in processing of fruit juices, and in baking; paper and pulp industry for de-inking of paper (Yassien *et al.*, 2014) along with some of the modern usage like production of biofuels like bioethanol, triphasicbiomethanation ; agricultural and plant waste management ; chiral separation and ligand binding studies (Gupta *et al*., 2012).

Various value added products can also be obtained from lignocellulosic wastes like reducing sugars, enzymes, biofuels, organic acids, compost, biocomposites, food and feed, medicines and biosorbents. To combat the competition between food and fuel, biomass can be utilized rather than food crops as an alternative for bio-alcohol production, where the cellulosic biomass from wastes, is being explored (Mtui, 2009).

Microbes provide useful molecules to digest cellulose and in this process commercially important by-product may be formed (Concoran *et al*.,). Our present work plan aims at isolation and characterization of a microbe / microbes that can break down the cellulose. The novelty of the work plan lies in our approach towards the bioremediation of various organic wastes like petha waste, paper, cloth and groundnut shells and yields some economically important end product.




BREAK DOWN OF CELLULOSE

## REVIEW OF LITERATURE

**Bryant (1959)** indicated the various types of microbes especially bacteria present in the rumen and also gave various media ingredients useful for culturing the microbes.

.**Xiao *et al.*, (2004)** described the standard Filter Paper Assay method to determine the cellulase activity.

**Kamra, (2005)** studied the microbial ecosystem of the rumen where different types of microbes acting on various substrates live synergistically with the host and work under different natural and feed associated constraints causing bio conversion of lignocellulosic biomass. **Bakare *et al.*, (2005)** purified Cellulase enzymes to apparent homogeneity from *Pseudomonas fluorescens* using various methodologies like ammonium sulphate precipitation, DEAE Sephadex A-50 ion chromatography exchange and Sephadex G-100 Gel filtration chromatography and PAGE. The enzymes so obtained were characterized by studying its various properties like Km values, optimum pH, optimum temperature and metal ion effect. **Mosier *et al.*, (2005)** described the physical and chemical pretreatment methods that remove the structural and compositional impediments in the cellulose hydrolysis process thereby increasing the enzyme hydrolysis rate.

**Corcoran *et al.*, (2008)** used dairy rumen microbes to produce cellulosic ethanol from sugar cane bagasse. The project focused specifically on hydrolysis step during which the cellulose is broken down to fermentable glucose. The optimal lab scale hydrolysis time period was found to be around 84 hours where the glucose produced could be efficiently be converted to ethanol, after which on further incubation these sugars were converted to volatile fatty acids which could not be then converted to ethanol. They performed emergy analysis and developed a large scale conceptual design system depending on the sustainability and efficiency of the lab scale system and predicted it to be more efficient than the existing processes of energy production.

**Kumar *et al.*, (2009)** mentions the use of various types' of recombinant microbes like *Saccharomyces cerevisiae* MT8-1, *Zymomonas mobilis* AX101, *Klebsiella oxytoca* P2 and *E.coli* KO11 for ethanol production after hydrolysis and found that thermophiles like *Themoanaerobacterium saccharolyticum* JW/SL-YS485 were also efficient. They were further able to ferment monomers like glucose, xylose, mannose, galactose and arabinose as well as polymers like xylan and hemicelluloses and hypothesized that as the presence of glucose causes enzymatic inhibition of cellulase, thus it is better to carry out saccharification and fermentation simultaneously. In the same year **Mtui, (2009)** highlighted various types of pretreatment methods and value added products being obtained from the lignocellulosic wastes.

**Khan *et al.*, (2011)**, isolated cellulase producing bacterial strain from cow dung. **AL-Rawi, (2011)** studied the production of Cellulase from rumen cellulolytic microorganisms.

**Verma *et al.*, (2012)** isolated cellulase from the microbe *Bacillus subtilis* from the soil samples. In the same year, **Das & Qin, (2012)** isolated and characterized different superior rumen microbes to find its application in animal feed stuff. Cellulase was isolated in its crude form and its activity accessed via two methods: Carboxymethyl Cellulose (CMC) activity & Filter Paper activity (FPA). **Gupta *et al.*, (2012)** similarly in the same year isolated eight cellulose degrading bacteria and determined their cellulolytic potential using various techniques like Congo red agar media assay, hydrolysis capacity, gravimetric analysis, DNS assay, FPA assay, etc.

**Javalkar & Alam, (2013)** generated microbial fuel cell and efficiently used bio-waste such as cow dung and cow's urine to generate electricity.

**Yassien *et al.*, (2014),** isolated cellulase producing *Streptomyces* strain C188 from the Saudi Arabia soil. Cellulase productivity was improved by modifying the media and then the enzymes were purified.

## OBJECTIVES OF RESEARCH

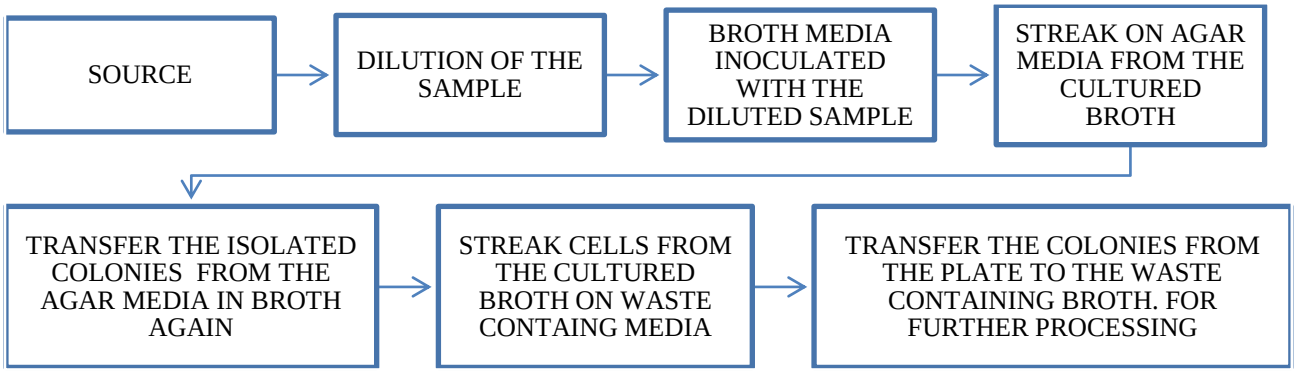
In the present plan of study microbes from Rumen fluid/ Urine/ Dung of ruminants (cow/ buffalo/ goat) will be isolated and thereby utilized for obtaining useful by product like biofuel from organic wastes. The following objectives shall be fulfilled:

1. Isolation, Characterization and Identification of Cellulase producing microbes.
2. Production and Extraction of Cellulase enzyme from the isolated microbes
3. Physico-chemical Characterization and Molecular analysis of the Cellulase enzyme
4. Treatment of different organic wastes to obtain useful byproduct.
5. Assessment of the obtained by-products.

## MATERIAL AND METHODS

1. Sample collection:- Rumen fluid/ Dung/ Urine from ruminants (Cow/ Buffalo/ goat)
2. Isolation, Characterization and Identification of Cellulase producing microbes:-
    - Isolation:- Specialized media ( Das & Qin, 2012) (Corcoran *et al* , 2008)

      -Cellulose containing media CMC media (Khan *et al.*, 2011)


SOURCE
DILUTION OF THE SAMPLE
BROTH MEDIA INOCULATED WITH THE DILUTED SAMPLE
STREAK ON AGAR MEDIA FROM THE CULTURED BROTH
TRANSFER THE ISOLATED COLONIES FROM THE AGAR MEDIA IN BROTH AGAIN
STREAK CELLS FROM THE CULTURED BROTH ON WASTE CONTAING MEDIA
TRANSFER THE COLONIES FROM THE PLATE TO THE WASTE CONTAINING BROTH. FOR FURTHER PROCESSING

    - Characterization: - Microscopic view, Congo red assay (Das & Qin, 2012), Filter paper assay (Xiao et al, 2004).
    - Identification:-Molecular characterization of the microbes-Genomic DNA isolation, PCR, BLAST and other bioinformatic techniques.
3. Action of microbes on Organic Wastes (sugarcane bagasse, paper, cloth {fiber}, groundnut shells and petha waste)( Corcoran *et al* , 2008).
4. Production of Cellulase enzyme using specific media and its Extraction - Electrophoresis and Chromatographic techniques (Yassien *et al*., 2014)
    - Physico-chemical Characterization of the obtained Cellulase enzyme (pH optima, temperature optima, etc) using - Bradford assay method (Bradford ,1976) and Dinito-salicylic acid (DNSA) method (Miller, 1959)(AL-Rawi, 2011)
    - Molecular Characterization of the Cellulase enzyme – SDS PAGE, 2D-Electrophoresis and Bioinformatic analysis.
5. Assessment will be done depending on the obtained by-product (alcohol, sugars, enzymes, etc) (Mtui, 2009).

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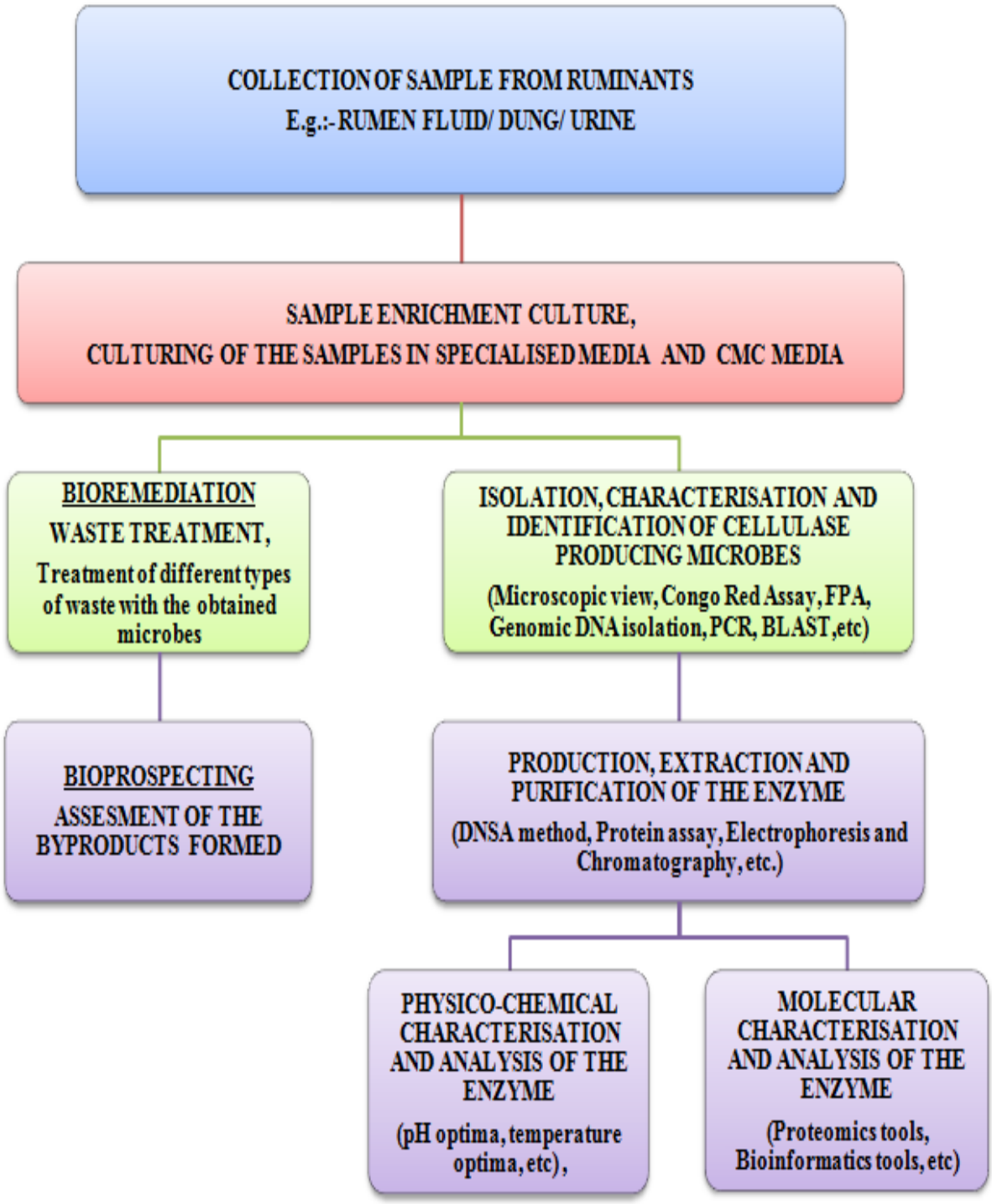
## PLAN OF WORK


COLLECTION OF SAMPLE FROM RUMINANTS
E.g.:- RUMEN FLUID/ DUNG/ URINE
SAMPLE ENRICHMENT CULTURE,
CULTURING OF THE SAMPLES IN SPECIALISED MEDIA AND CMC MEDIA
BIOREMEDIATION
WASTE TREATMENT,
Treatment of different types of waste with the obtained microbes
ISOLATION, CHARACTERISATION AND IDENTIFICATION OF CELLULASE PRODUCING MICROBES
(Microscopic view, Congo Red Assay, FPA, Genomic DNA isolation, PCR, BLAST, etc)
BIOPROSPECTING
ASSESMENT OF THE BYPRODUCTS FORMED
PRODUCTION, EXTRACTION AND PURIFICATION OF THE ENZYME
(DNSA method, Protein assay, Electrophoresis and Chromatography, etc.)
PHYSICO-CHEMICAL CHARACTERISATION AND ANALYSIS OF THE ENZYME
(pH optima, temperature optima, etc),
MOLECULAR CHARACTERISATION AND ANALYSIS OF THE ENZYME
(Proteomics tools, Bioinformatics tools, etc)